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ORIGINAL ARTICLE

Bacterial-biota dynamics of eight bryophyte species from different ecosystems

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Abstract Despite the importance of bryophyte-associated microorganisms in various ecological aspects including their crucial roles in the soil-enrichment of organic mass and N_2 fixation, nonetheless, little is known about the microbial diversity of the bryophyte phyllospheres (epi-/endophytes). To get insights into bacterial community structures and their dynamics on the bryophyte habitats in different ecosystems and their potential biological roles, we utilized the 16S rRNA gene PCR-DGGE and subsequent phylogenetic analyses to investigate the bacterial community of eight bryophyte species collected from three distinct ecosystems from western Japan. Forty-two bacterial species belonging to *γ-proteobacteria* and *Firmicutes* with 71.4% and 28.6%, respectively, were identified among 90 DGGE gel band population. These DGGE-bands were assigned to 13 different genera with obvious predomination the genus *Clostridium* with 21.4% from the total bacterial community. These analyses provide new insights into bryophyte-associated bacteria and their relations to the ecosystems.

1. Introduction

Bryophytes, a group of lower non-vascular plants that is composed of *Musci* (mosses), *Hepaticae* (liverworts), and *Anthrocerotae* (hornworts), have been taxonomically placed between the algae and the *pteridophytes*, as first terrestrial plants (Kenrick and Crane, 1997; Edwards et al., 1995). This group entails more than 10,000 species and inhabits a diversity of ecosystems ranging from fresh water sponges in the tropics to the caribou dung patches of the arctic tundra region (Pharo and Zartman, 2007). Bryophytes have been suggested as excellent candidates for appraising the ecological and evolutionary impacts of the habitat fragmentation due to their global ubiquity, fast-growing nature, substrate specificity, and dominant haploid gametophytes (Pharo and Zartman, 2007). They have also been adopted and employed as model organisms and harnessed for different kinds of biotechnological applications (Oliver et al., 2000; Decker et al., 2003). And due to their ability for fast growth, water maintenance and drought tolerance, the utilization of bryophytes as green-roof is growing astoundingly growth (Tani et al., 2011). Previous

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reports have also suggested possible usages for bryophytes as bio-monitors, and as an important factor to document the atmospheric chemistry as well as their importance in the biogeochemical processes (Turetsky, 2003; Cornelissen et al., 2007), and their impact in the ecosystems at large scales (Rochefort, 2000; Nilsson and Wardle, 2005; Crowly and Bedford, 2011).

These unique properties have turned eyes toward the bryophyte group in order to better exploit it in different aspects. Ecologically, bryophytes colonize unique and microbial-favorable niches, which are supposed to be densely occupied with a huge diversity of microorganisms, but hitherto little is known about the nature and bio-functional diversity of these microbial communities compared with respect to their diverse importance at different aspects (Crowly and Bedford, 2011). This makes the study of bryophyte-associated microorganisms and the understanding of their roles as co-exists with the environment especially interesting.

Despite the efforts that have been paid to investigate the microbial structures of different eco-systems including aqueous, terrestrial, soil animal and plant systems, there is still shortage and little attention regarding this group of important climatic terrestrial plants (Hornschuh et al., 2002). Apart from this, some genera such as *Sphagnum* have been studied thoroughly to understand their microbial community structure and associated biological importance (Hornschuh et al., 2002; Bragina et al., 2013), however, the knowledge on the prevalence and diversity of the bryophyte epi-/endophyte microorganisms remain scarce. Microbial strains such as *Burkholderia*, *Serratia*, *Hafnia*, *Pantoea*, *Methanobacteria* and *Methylobacteria* were found abundantly as endophytes, epiphytes or both in some mosses (Bragina et al., 2013; Opelt and Berg, 2004). Interestingly, some of these microorganisms have been demonstrated to possess the ability in producing plant-growth regulators (PGRs), which is an important characteristic for bio-fertilizing applications and might somehow explain their roles in the moss growth (Hornschuh et al., 2002; Tani et al., 2011). The ability of these moss-associated bacteria in PGR production and their possible interactions with plant tissues during growth are of high interest (Opelt and Berg, 2004). In contrast, other reports have shown that many of these isolated moss-associated bacteria have been demonstrated to possess different antagonistic properties, such as *Pseudomonas putida*, *Xanthomonas* sp., *Serratia* sp., and *Bacillus* sp. (Opelt et al., 2007).

The interaction between the diazotrophic and non-diazotrophic bacterial groups in non-leguminous plants and their roles in such interaction have been previously suggested and named ANFICO, the anaerobic nitrogen-fixing consortium (Minamisawa et al., 2004). Interestingly, in our preliminary screening of bacteria associated with mosses collected from different regions in Japan, we noticed similar predomination of diazotrophic and non-diazotrophic microorganisms that drives us for further investigation on the moss associated microorganisms.

In this study, we screened the microbial diversity of eight bryophyte species using the culture-independent approach of PCR-DGGE based on the divergent regions of the 16S rRNA gene (Muyzer et al., 1993). The relationship between the ecosystem and bryophyte species in association with bacterial community structure fluctuations was discussed and further questions were opened for future researches. Nevertheless, this report should be treated as a preliminary study and further investigation including additional factors such as seasonal changes, more diverse ecosystems and associated mosses is required for a comprehensive and critical evaluation.

2. Materials and methods

2.1. Sampling sites and strategy

To evaluate the bacterial community structure of the bryophytes, gametophytes of nine bryophytes (representative of eight distinct species) were collected during the spring season of 2009 from the Kurashiki city, which is located in western part of Japan with Latitude: N34.58° 35′ and Longitude: E133.77° 46′. Three different sites were purposely targeted for sampling; (a) Kurashiki Ivy Square museum, a region that receives hundreds of visitors weekly (designated as highly populated soil), (b) Tsurugatayama Hill (virgin wet rocks), in

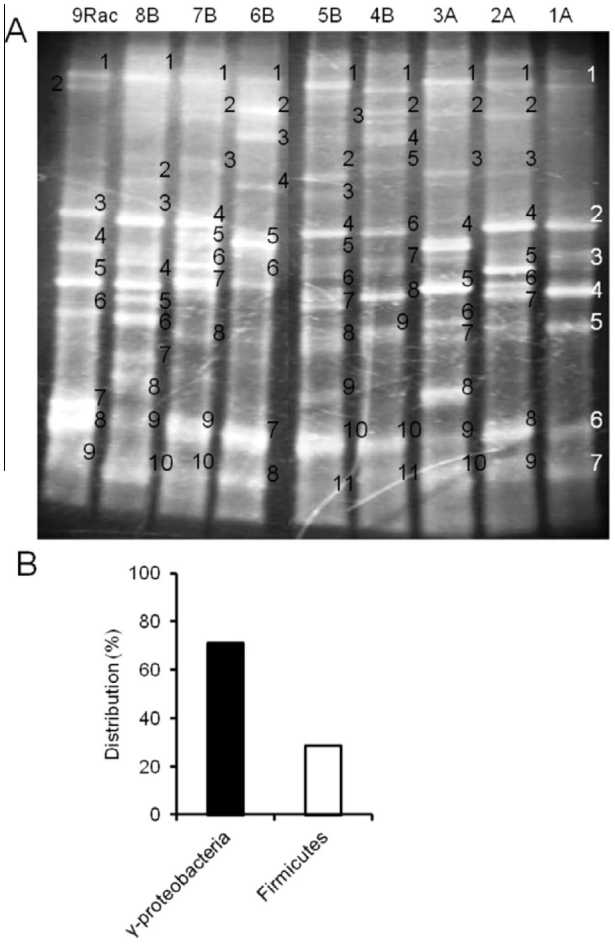

Figure 1 PCR-DGGE gel of bryophytes associated bacteria and the taxonomy distribution. (A) 16S rRNA gene PCR-DGGE characterization of nine bryophyte associated bacteria of three different ecosystems. Letters A, B and Rac symbols indicated the samples of highly populated-soil (intact), virgin-rocks and *Racomitrium* moss isolated from managed soil (green roof), respectively. (B) The taxonomical distribution percentage of the bacterial community isolated from nine bryophytes of different ecosystems.

Table 1 Phylogenetic analysis of the bacterial community structure of eight bryophyte species isolated from three different ecosystems of the Kurashiki city, Western-Japan.

Closest BLAST matches	Band	Ecosystems	Bryophyte source	Closest matches characterization		
				Accession no.	Identity (%)	Taxonomic group
Citrobacter murliniae (*T*)	1A3	Intact soil (populated)	*Haplocladium microphyllum*	AF025369	93	*γ-Proteobacteria*
Klebsiella terrigena strain ATCC 33257T	1A4	Intact soil (populated)	*Haplocladium microphyllum*	AF129442	88	*γ-Proteobacteria*
Ps. fluorescens; *Ob. Proteus*; *Kl. intermedia*	2A1	Intact soil (populated)	*Brachythecium buchananii*	–	98	*γ-Proteobacteria*
Kl. intermedia; *Enterobacter* sp.; *Hafnia* sp.	2A2	Intact soil (populated)	*Brachythecium buchananii*	–	98	*γ-Proteobacteria*
Kl. intermedia; *Enterobacter* sp.; *Hafnia* sp.	2A3	Intact soil (populated)	*Brachythecium buchananii*	–	100	*γ-Proteobacteria*
Ob. proteus; *Hafnia* sp.; *Enterobacter* sp.	2A4	Intact soil (populated)	*Brachythecium buchananii*	–	100	*γ-Proteobacteria*
Kl. intermedia; *Pa. citrea*; *Enterobacter* sp.	2A5	Intact soil (populated)	*Brachythecium buchananii*	–	98	*γ-Proteobacteria*
Clostridium butyricum	2A6	Intact soil (populated)	*Brachythecium buchananii*	–	96	*Firmicutes*
Clostridium puniceum	2A7	Intact soil (populated)	*Brachythecium buchananii*	–	100	*Firmicutes*
Pectobacterium wasabiae ATCC 43316	3A1	Intact soil (populated)	*Trachycystis microphylla*	U80199	81	*γ-Proteobacteria*
Pectobacterium betavasculorum ATCC 43762	3A3	Intact soil (populated)	*Trachycystis microphylla*	U80198	82	*γ-Proteobacteria*
Dickeya dieffenbachiae CFBP 2051	3A4	Intact soil (populated)	*Trachycystis microphylla*	AF520712	80	*γ-Proteobacteria*
Serratia proteamaculans DSM 4543	3A5	Intact soil (populated)	*Trachycystis microphylla*	AJ233434	96	*γ-Proteobacteria*
Serratia proteamaculans DSM 4543	3A6	Intact soil (populated)	*Trachycystis microphylla*	AJ233434	91	*γ-Proteobacteria*
Serratia grimesii DSM 30063	3A7	Intact soil (populated)	*Trachycystis microphylla*	AJ233430	91	*γ-Proteobacteria*
Serratia proteamaculans DSM 4543	3A8	Intact soil (populated)	*Trachycystis microphylla*	AJ233434	96	*γ-Proteobacteria*
Klebsiella oxytoca ATCC13182T	3A10	Intact soil (populated)	*Trachycystis microphylla*	Y17655	94	*γ-Proteobacteria*
Salmonella enterica subsp. Enterica ATCC 13311	4B1	Virgin rocks	*Brachythecium plumosum*	X80681	90	*γ-Proteobacteria*
Anaerobacter polyendosporus DSM 5272	4B5	Virgin rocks	*Brachythecium plumosum*	Y18189	92	*Firmicutes*
Citrobacter freundii DSM 30039	5B1	Virgin rocks	*Bryum* sp.	AJ233408	87	*γ-Proteobacteria*
Enterobacter cowanii CIP 107300	5B2	Virgin rocks	*Bryum* sp.	AJ508303	86	*γ-Proteobacteria*
Buttiauxella warmboldiae DSM 9404	5B4	Virgin rocks	*Bryum* sp.	AJ233406	96	*γ-Proteobacteria*
Clostridium chartatabidum DSM 5482	5B7	Virgin rocks	*Bryum* sp.	X71850	77	*Firmicutes*
Pseudomonas antarctica CMS 35	6B1	Virgin rocks	*Hypnum plumaeforme*	AJ537601	93	*γ-Proteobacteria*
Pseudomonas cedrina CFML 96-198	6B2	Virgin rocks	*Hypnum plumaeforme*	AF064461	83	*γ-Proteobacteria*
Anaerobacter polyendosporus DSM 5272	6B3	Virgin rocks	*Hypnum plumaeforme*	Y18189	91	*Firmicutes*
Anaerobacter polyendosporus DSM 5272	6B4	Virgin rocks	*Hypnum plumaeforme*	Y18189	84	*Firmicutes*
Clostridium disporicum DSM 5521	6B6	Virgin rocks	*Hypnum plumaeforme*	Y18176	95	*Firmicutes*
Clostridium saccharoperbutylacetonicum N1-4	6B7	Virgin rocks	*Hypnum plumaeforme*	U16122	92	*Firmicutes*
Citrobacter murliniae (T)	1A3	Intact soil (populated)	*Haplocladium microphyllum*	AF025369	93	*γ-Proteobacteria*
Klebsiella terrigena strain ATCC 33257T	1A4	Intact soil (populated)	*Haplocladium microphyllum*	AF129442	88	*γ-Proteobacteria*
Buttiauxella warmboldiae DSM 9404	7B2	Virgin rocks	*Trachycystis microphylla*	AJ233406	100	*γ-Proteobacteria*
Serratia proteamaculans DSM 4543	7B3	Virgin rocks	*Trachycystis microphylla*	AJ233434	84	*γ-Proteobacteria*
Buttiauxella ferragutiae DSM 9390	7B4	Virgin rocks	*Trachycystis microphylla*	AJ233402	100	*γ-Proteobacteria*
Enterobacter asburiae JCM6051	7B5	Virgin rocks	*Trachycystis microphylla*	AB004744	100	*γ-Proteobacteria*
Clostridium saccharoperbutylacetonicum N1-4	7B6	Virgin rocks	*Trachycystis microphylla*	U16122	94	*Firmicutes*
Erwinia rhapontici ATCC 29283	8B1	Virgin rocks	*Reboulia hemisphaerica sub. orientalis*	U80206	88	*γ-Proteobacteria*
Citrobacter murliniae CDC 2970-59	8B2	Virgin rocks	*Reboulia hemisphaerica sub. orientalis*	AF025369	92	*γ-Proteobacteria*
Pantoea ananatis ATCC 33244	8B3	Virgin rocks	*Reboulia hemisphaerica sub. orientalis*	U80196	96	*γ-Proteobacteria*
Citrobacter murliniae CDC 2970-59	8B4	Virgin rocks	*Reboulia hemisphaerica sub. orientalis*	AF025369	77	*γ-Proteobacteria*
Pantoea ananatis ATCC 33244	8B5	Virgin rocks	*Reboulia hemisphaerica sub. orientalis*	U80196	93	*γ-Proteobacteria*
Clostridium saccharoperbutylacetonicum N1-4	9Rac2	Managed soil	*Racomitrium japonicum*	U16122	97	*Firmicutes*
Clostridium puniceum DSM 2619	9Rac4	Managed soil	*Racomitrium japonicum*	X71857	89	*Firmicutes*
Clostridium puniceum DSM 2619	9Rac5	Managed soil	*Racomitrium japonicum*	X71857	93	*Firmicutes*
Buttiauxella warmboldiae DSM 9404	7B2	Virgin rocks	*Trachycystis microphylla*	AJ233406	100	*γ-Proteobacteria*

which four different bryophyte species were collected from rock walls and not from the surface in order to avoid any possibility of human/animal intervention, and (c) the green roof of the Institute for Plant Science and Resources of the Okayama University (Managed Soil). These sampling areas were chosen based on our basic hypothesis that implies critical differences in the intactness of the soil. Therefore, the bryophyte diversity was somewhat limited in some ecosystem, particularly the managed-soil area, where it was only possible to collect the *Racomitrium japonicum* moss, since it is the only moss that was utilized for the roof-greening at the time of sampling.

2.2. Bryophyte taxonomy

Bryophytes were authenticated as eight taxonomically distinct species at the Department of life sciences at the Okayama University of Science. These bryophytes were categorized into three groups based on the ecosystem; (a) highly-populated soil, which includes *Haplocladium microphyllum*, *Brachythecium buchananii*, and *Trachycystis microphylla*, (b) virgin wet rocks include *Brachythecium plumosum*, *Bryum* sp., *Hypnum plumaeforme*, *T. microphylla*, and *Reboulia hemisphaerica* sub. *Orientalis*, and (c) managed-soil which includes one species, *R. japonicum* (Rac). *T. microphylla* was the only species that was found dominant in two distinct habitats.

2.3. Total DNA extraction and PCR amplification

A simple protocol for total DNA extraction was used to prepare the total genomic DNA for DGGE-PCR analyses (see Tani et al., 2011). A total of 0.5 g of room-temperature dried bryophyte (gametophytes) was measured and cleaned carefully from the soil particles and washed at least twice before transferred into a sterile 50 ml Falcon-tube filled with autoclaved ddH_2O. The samples were shaken vigorously before incubating overnight at room-temperature (18.0 ± 2 °C). In the following day the total community DNA of different samples was isolated from the supernatants. These gametophytes were selected from at least three independently collected mosses of each species belonging to the same ecosystem. The DNA isolation was done using SNET buffer composed of 20 mM Tris–HCl, pH 8.0, 5 mM EDTA, 400 mM NaCl, 0.3% SDS, and 20 µm/ml proteinase K and the resulted DNA was used directly for the PCR reaction (Tani et al., 2011).

2.4. DGGE, 16S rRNA and phylogenetic analysis

Amplicons of the variable regions of bacterial 16S rRNA gene were analyzed by DGGE using the universal 16S rRNA primers, forward V3 region of *Escherichia coli* (3′-ACTCC TACGGGAGGCAGCAG-5′), and reverse (*E. coli* positions 517–534: 5′-ATTACCGCGGCTGCTGG-3′). The forward primer (5′ prime end) was labeled with [5′-CGCCCGCCGCG CGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTA-CGGGAGGCAGCCAG-3′] as GC-clamp to prevent the complete melting of the PCR products during fragment separation in a denaturing gradient of the DGGE (Muyzer et al., 1993; Tani et al., 2012). The PCR was performed using a NovaTaq DNA polymerase (Shimadzu, Kyoto, Japan). The PCR products were separated in a 40–60% denaturing gradient gel of 7 M urea and 40% (v/v) formamide at 60 °C using the DCode machine (Bio-Rad, Tokyo, Japan). DGGE gel bands were analyzed using a visual gel analysis software package, of which about 90 prominent bands were excised from the DGGE gel and re-amplified with the same primers, but the forward primer has no GC-clamp (Fig. 1A). The DNA products were then purified for DNA sequencing using a MagExtractor kit following manufacturer's instruction. The DNA sequences were analyzed for the closest neighbors among sequenced 16 rDNA regions of different bands using the EzTaxon server (Chun et al., 2007).

3. Results and discussion

The 16S rRNA based DGGE method has been used widely to investigate the microbial community structures of different ecosystems ranging from oceans to small niches in mite guts

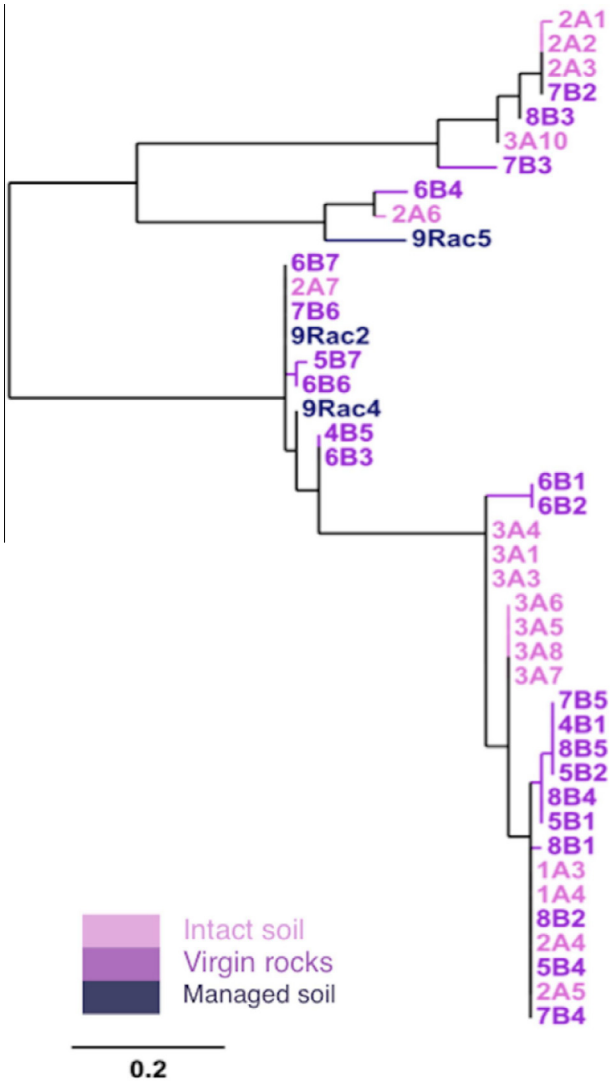

Figure 2 Neighbor-joining Phylogenetic analysis constructed based on comparative analysis of 42 individual amplified 16S rRNA gene of the bacterial community structure associated with the bryophyte species using multiple alignments ClustalW. Scale represents dis-similarity percentage. Bands labels are indicated in Table 1.

as well as in bryophyte phyllospheres (Muyzer et al., 1993). In this study, 42 out of 90 excised dominant PCR-DGGE gel bands from eight bryophyte species were successfully assigned (Fig. 1A and Table 1). The phylogenetic analyses indicated predomination of two major taxa, *γ-proteobacteria* and the *Firmicutes*, which colonized the bryophytes with 71.4% and 28.6%, respectively (Fig. 1B and Table 1). The results showed a similar distribution of these bacterial groups in intact soil and virgin rock, whereas low diversity was found in the *R. japonicum* from the managed soil (Rac), which was predominated mainly by the genus *Clostridium*, which is reasonable since only nine bands were excised from *R. japonicum*, of which only three bands were sequenced and analyzed due to a contamination issue. This is in agreement with the previous study (Tani et al., 2012), which showed a predomination of *Firmicutes* on intact *R. japonicum* cultivated on managed-soil, while the same study has indicated a change in the predominance when cultivating this moss in an *in vitro* liquid culture (Tani et al., 2012). The change in predominance upon ecosystem shift was also reported in the microbial community structure of the foraged-fed cattle rumens (Pitta et al., 2010).

Although, differences in the community structures were noticed at the species level regardless of the ecosystem nature, some bacterial genera had similar distribution among different ecosystems. The *γ-proteobacteria* genera such as *Klebsiella*, *Obesumbacterium*, *Pectobacterium* and *Dickeya* were found exclusively associated with the bryophytes of the highly populated-soil habitats (Fig. 2 and Table 1). In contrast, the virgin-rock bryophytes were colonized with both *Firmicutes* and *γ-proteobacteria*, of which six genera *Salmonella*, *Enterobacter*, *Buttiauxella*, *Pseudomonas*, *Erwinia*, and *Pantoea* are from the *γ-proteobacteria* group, while only two genera *Anaerobacter* and *Clostridium* belong to the *Firmicutes*. On the other hand, genera like *Citrobacter*, *Pseudomonas*, *Clostridium* and *Serratia* were found common among highly-populated soil and virgin-rocks associated bryophytes and are not affected by changes in the ecosystem but not the host species, whereas, genera such as *Buttiauxella*, *Erwinia*, *Pantoea* and *Anaerobacter* were found limited to the virgin-rocks associated bryophytes. In contrast, the genus *Clostridium* was found to colonize all of the bryophyte species and not influenced by the changes in the ecosystem. The managed-soil related *R. japonicum* deemed to be less diverse in terms of bacterial diversity with apparent predomination for the genus *Clostridium*. It should be mentioned here that the *R. japonicum* has indicated clear shifting on its microbial community when transferred into liquid controlled hydroponic culture, which indicates that the predomination for *Clostridium* is more likely related to the ecosystem changes but not species-dependent (Tani et al., 2012).

As shown in Table 1, the bryophyte species that were sampled from the same ecosystem have shown differences in their bacterial community structures, which might indicate a host-dependent microbial community dynamics phenomenon in agreement with the previous report (Bragina et al., 2012). Although, other factors such as ecosystem impacts should not be ruled out taking into account the limited resources employed in the current study. It has been observed that during the sampling of this study there was no matching among the collected bryophytes of different ecosystems, except the genus *T. microphylla*, which was sampled from both highly-populated soil and virgin-rocks (see Materials and Methods). Comparison of the bacterial community revealed that the genus *T. microphylla* of the highly-populated soil was found to be colonized by one phylogenetically related group, *γ-proteobacteria*, with a predomination for the genus *Serratia* (50%), while that of the virgin-rocks was colonized by both *γ-proteobacteria* (80%) and *Firmicutes* (20%) with obvious predomination the genus *Buttiauxella* (40%) (Fig. 3). The genus *Buttiauxella* was also found to colonize another species that is *Bryum* sp., whereas the analyses indicated its absence from

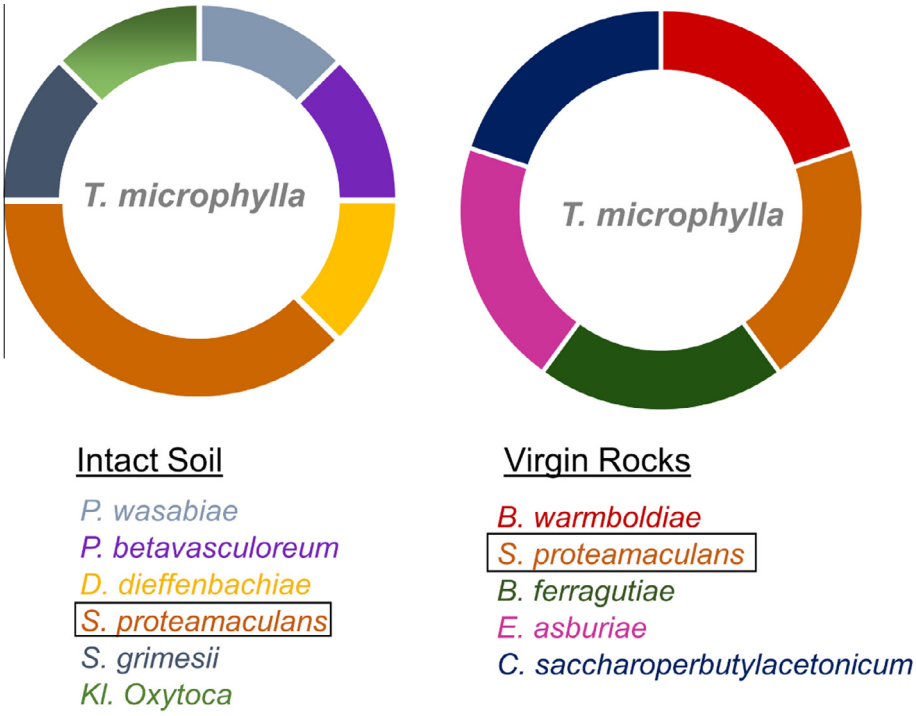

Figure 3 Ecosystem distribution of *T. microphylla* associated bacteria.

other ecosystems. In addition to the host-dependency, this finding can be attributed to the bacterial community fluctuations among different ecosystems (Tani et al., 2012; Pitta et al., 2010). The results have also partly agreed with a recent study by Bragina et al. (2012), which indicated that bacterial community structures are highly specific to their mosses. In fact this is a well-known phenomenon, i.e., the impact of the plant species on the microbial community structures (Berge et al., 2006). Whereas, the dependency of the bacterial community structures on the specific ecosystem, which has also been shown in this study needs further evaluation, since most of the bacteria tend to distribute independently with respect to the ecosystem and/or bryophyte species.

The results indicate the predomination of the *Firmicutes* in the managed-soil which is in agreement with Tani et al. (2012), although it is difficult from the current data to attribute this domination to the shift in the ecosystem since our sampling did not include *R. japonicum* from any ecosystem other than that of managed-soil. Some of the assigned bacteria of this report are well known as plant-associated bacteria for their impact on the plant viability in different ways either by promoting the growth such as diazotrophic and non-diazotrophic (*Clostridium* sp. and *Enterobacter* sp.) bacterial consortium (Minamisawa et al., 2004) or by causing different kinds of damaging diseases such as the well-known pathogenic bacterium, *Pantoea ananatis* (Opelt and Berg, 2004). Two *Serratia* species; *Serratia proteamaculans*, and *Serratia liquefaciens* found predominantly in the mosses, *Sphagnum* and *Aulacomnium* have been demonstrated to possess the most effective antagonistic properties among many other bacterial isolates from the same mosses (Opelt and Berg, 2004). In the current study, the genus *Serratia*, e.g., *S. proteamaculans*, was found exclusively in the moss *T. microphylla* of both virgin-rocks and highly-populated soils (Fig. 3), which might indicate species-specificity of this genus of biological importance. The colonization of bryophytes with *Clostridia* was previously observed to co-exist with other *Firmicutes* such as *Bacillus* sp. (*data not shown*), which has drawn our attention to the ANFICO phenomenon found in the non-leguminous plants, where the aerobic non-diazotrophic bacteria coat the N_2-fixing *Clostridia* and eliminate the oxygen to enable them to fixate N_2 (Minamisawa et al., 2004). The ANFICO phenomenon is of interest for N_2-fixation application in non-leguminous plants such as various gramineous cereal crops.

4. Conclusion

We report the bacterial community structures of eight bryophyte species collected from three distinct ecosystems. Data indicate the domination of the genus *Clostridium* over other bacterial species. The bacterial communities detected in the bryophytes collected from the highly populated-soil (un-treated) and virgin-rocks were highly diverse when compared to the managed-soils. Further studies on the biological importance as well as the fluctuations of these bacterial communities over different ecosystems are underway.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

Acknowledgments

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